Carboplatin-induced upregulation of pan β-tubulin and class III β-tubulin is implicated in acquired resistance and cross-resistance of ovarian cancer

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Abstract
Resistance to platinum- and taxane-based chemotherapy represents a major obstacle to long-term survival in ovarian cancer (OC) patients. Here, we studied the interplay between acquired carboplatin (CBP) resistance by using two OC cell models, MES-OV CBP and SK-OV-3 CBP, and non-P-glycoprotein-mediated cross-resistance to paclitaxel (TAX) observed only in MES-OV CBP cells. Decreased platination, mesenchymal-like phenotype, and increased expression of α- and γ-tubulin were observed in both drug-resistant variants compared with parental cells. Both variants revealed increased protein expression of class III β-tubulin (TUBB3) but differences in TUBB3 branching and nuclear morphology. Transient silencing of *TUBB3* sensitized MES-OV CBP cells to TAX, and surprisingly also to CBP. This phenomenon was not observed in the SK-OV-3 CBP variant, probably due to the compensation by other β-tubulin isotypes. Reduced TUBB3 levels in MES-OV CBP cells affected DNA repair protein trafficking and increased whole-cell platination level. Furthermore, TUBB3 depletion augmented therapeutic efficiency in additional OC cells, showing *vice versa* drug resistant pattern, lacking β-tubulin isotype compensation visible at the level of total β-tubulin (TUBB) *in vitro* and *ex vivo*. In summary, the level of TUBB in OC should be considered together with TUBB3 in therapy response prediction.

Keywords: cancer drug resistance, ovarian cancer therapy, microtubules, cellular trafficking, Pt-drugs accumulation

Introduction

Most epithelial ovarian cancer (OC) patients are diagnosed with advanced-stage disease due to the late appearance of symptoms and lack of early diagnostic biomarkers. Despite tremendous progress regarding targeted therapy and immunotherapy, the current treatment of OC remains cytoreductive surgery followed by platinum- and taxane-based chemotherapy [1]. Platinum drugs such as carboplatin (CBP) induce various damages in cells, but it is broadly accepted that their binding to DNA causes the most toxic lesions [2]. The cytotoxicity of taxanes, such as paclitaxel (TAX), is based on the binding and stabilization of polymerized microtubules. Due to their different modes of action, CBP and TAX are often combined in cancer therapy [3]. However, the success of chemotherapy is often challenged by acquired or intrinsic drug resistance. In fact, despite the initial positive clinical response to chemotherapy, up to 80% of OC patients will eventually relapse, become platinum/taxane unresponsive, and develop metastases [1]. Epithelial-mesenchymal transition (EMT), a process paramount for metastasis, where tumor epithelial cells lose their cell polarity and cell–cell adhesion and gain migratory and invasive properties of metastatic cells, is associated with the development of drug resistance [4], although it is still not known exactly how these two phenomena are interconnected [5].

Different molecular mechanisms are involved in platinum and taxane drug resistance [3]. Except for cell death regulators [6], resistance mechanisms induced by platinum [7] or by taxane drugs [8] do not overlap. To date, most publications have focused on a separate investigation of these two processes, not considering the possible linkage between them. Previously, we have shown that multidrug resistance-independent (non-MDR) paclitaxel-resistant OC OVCAR-3/TP GFP and MES-OV/TP GFP cell lines are cross-resistant to CBP and display a mesenchymal-like phenotype and an increased level of class III β-tubulin (TUBB3), which correlates with decreased expression of miR-200 family members, especially miR-200c and miR-141 [9]. At that point, we did not further explore the signaling pathway(s) behind CBP cross-resistance in more detail. Only a few groups investigated cross-resistance to taxanes in platinum-resistant cell lines. Notably, the cisplatin (cDDP)-resistant OC IGROVCDDP cell line was shown to be cross-resistant to paclitaxel due to overexpression of P-glycoprotein [10]. Likewise, cDDP-induced NF-κB activation through tumor necrosis factor signaling is thought to contribute to the cross-resistance to anti-microtubule drugs in the OVCAR-8 cell line model [11]. Stordal et al. (2007 and 2012) found that 17% of established cancer cells with acquired resistance to cDDP were cross-resistant to TAX [3, 10], 41% were not cross-resistant and 28% became hypersensitive to paclitaxel, suggesting that the majority of cancer patients might benefit from receiving chemotherapy that alternates between cisplatin and paclitaxel treatment. They underlined that the challenge is to identify the patients who will respond well to alternating therapy [10]. To this end, it is essential to identify possible intersectional molecular mechanisms/targets of dual resistance and to explore them as possible predictive biomarkers.

Here, we aimed to investigate the carboplatin-induced paclitaxel cross-resistance that occurs only in some cellular models, its correlation with CBP-induced TUBB3 expression, and the possible use of our findings to predict therapeutic response. For this purpose, we established OC cell line models characterized by clinically relevant acquired resistance to CBP, a mesenchymal-like phenotype, and different responsiveness to taxane treatment.

Materials and Methods

Chemicals and antibodies

Carboplatin was purchased from Sigma–Aldrich (St. Louis, USA), dissolved in water, and kept at –20 °C. Paclitaxel was obtained from the drug repository of the National Cancer Institute (Bethesda, USA), dissolved in pure ethanol, and kept at –20 °C. Resazurin was purchased from Sigma–Aldrich, dissolved in NaCl/Pi buffer, and kept at 4 °C. MK571 (Sigma–Aldrich) was dissolved in water and kept at –20 °C. 5-Aza-2′-deoxycytidine (5-aza; Sigma–Aldrich) was dissolved in acetic acid:water (1:1) and kept at –20 °C. The antibodies used are listed in Table S1. Ponceau S (Sigma–Aldrich) was dissolved in water and kept at room temperature.

Cell lines

Human ovarian adenocarcinoma MES-OV and SK-OV-3 cell lines were obtained from a cell culture bank (ATCC, Manassas, USA). Human ovarian carcinoma variants resistant to CBP (MES-OV CBP and SK-OV-3 CBP) were selected by drug treatment of parental cells as described previously [12]. MES-OV/TP and GFP-expressing OVCAR-3/TP variants were established as described previously [9, 13]. Cell models #6 and #8 were isolated from ascites of randomly selected either untreated (#6) or paclitaxel-treated (#8) nude mice injected intraperitoneally with the OVCAR-3 TP GFP cell line [13]. GFP-positive cells were sorted twice. All cell lines were grown in McCoy's medium supplemented with 10% fetal bovine serum (Gibco/Thermo Fisher Scientific, Waltham, USA) and cultured in a humidified atmosphere of 5% CO_2 at 37 °C. Authentication of human cell lines was performed by STR DNA profiling analysis (Microsynth AG, Balgach, Switzerland).

Determination of cell viability

Cell viability was determined using the Alamar Blue colorimetric assay [14]. The absorbance was measured using a microplate reader (Tecan, Männerdorf, Switzerland). The mass survival assay was used to evaluate cell survival following treatment with the epigenetic inhibitor 5-Aza-2′-deoxycytidine and CBP. The cells were fixed with cold methanol and stained with crystal violet. ImageJ (National Institutes of Health, Bethesda, USA) was used to measure the intensity of the color and area under the curve (AUC), proportional to the surface covered by the attached cells.

Determination of gene and miRNA expression by real-time PCR (qPCR)

Total RNA was isolated from subconfluent growing cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). One μg of RNA and a RevertAid First Strand cDNA Synthesis Kit (Thermo Fischer Scientific) or miScript II RNA Kit (Qiagen) was used for first-

strand cDNA synthesis from mRNA or miRNA respectively. For measurement of gene or miRNA expression, 1× SYBR® Universal PCR Master Mix (Applied Biosystems/Thermo Fisher Scientific) was used according to the manufacturer's instructions, with *GAPDH* or *RNU6B* as an internal control respectively. The primer sequences used for gene expression analysis are listed in Table S2, while miScript Primer Assays (Qiagen) were used to detect miRNA family members.

All real-time PCRs were run in the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems/Thermo Fisher Scientific). The amplification efficiency was determined by performing dilution series experiments. Dissociation curves were recorded after each run to confirm primer specificity. All reactions were performed in triplicate.

Determination of whole-cell and DNA platination

To determine whole-cell platination, a previously described protocol was used [15]. Briefly, upon drug treatment cells were rinsed with ice-cold PBS and harvested into 10 mL of ice-cold PBS with a rubber policeman. After centrifugation, the cells were resuspended in PBS, an aliquot was used for determination of the cell number, and the remainder was digested in 70% nitric acid. Cell lysates were heated for 2.5 h at 65 °C, diluted to 5% nitric acid, and assayed for platinum content. To detect DNA platination DNA was isolated using DNeasy Blood & Tissue Kits (Qiagen). The concentration of isolated DNA was measured photometrically, and the amount of platinum (Pt) atoms bound to DNA was calculated. All platination measurements were performed by high-resolution inductively coupled plasma–mass spectrometry (HR ICP–MS) using Element 2 (Thermo Finnigan, Bremen, Germany). Calibration standards were prepared using platinum standard solutions (1 μg L^{-1}, Atomic Spectroscopy Standard Solution, Fluka, Buchs, Switzerland). Indium (1 μg L^{-1}, Indium Atomic Spectroscopy Standard Solution, Fluka) was added to all solutions as an internal standard. The platinum-cell content was calculated using the relative molar mass of Pt and the cell number was determined for each sample. The platinum-nucleotide content was calculated using the relative molar masses of Pt and nucleotides.

Determination of protein expression by Western blot analysis

Protein expression was determined by Western blot analyses. Cells were trypsinized and harvested by centrifugation, washed with PBS, and resuspended in sonication buffer (20 mM Tris/HCl, pH 8.5, 1 mM EDTA, 5% glycerin, 1 mM DTT, 0.5 mM PMSF). After sonication (Cole-Parmer 130-Watt Ultrasonic Processors, Cole-Parmer, Vernon Hills, USA), cell debris was removed by centrifugation (15 min, 20,000 x *g* at 4 °C). The supernatants containing total cellular proteins were collected, and the protein concentration was determined (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific). Thirty micrograms of total cellular proteins was loaded onto 7.5-15% SDS polyacrylamide gels and run for two hours at 35 mA (Bio-Rad, Hercules, USA). Separated proteins were transferred onto a 0.2-μm nitrocellulose membrane (Trans-Blot Turbo Midi Nitrocellulose Transfer Packs; Bio-Rad) using a Trans-Blot Turbo Transfer System (Bio-Rad). Membrane was incubated in blocking buffer (5% nonfat dry milk in 0.1% Tween 20 in TBS (TBS-T)) for 1 h at room temperature. Incubation with monoclonal antibodies was performed overnight at 4 °C, followed by two-hour incubation with host species-specific horseradish peroxidase-conjugated secondary polyclonal antibodies at room temperature. After washing with TBS-T, proteins were visualized with ECL (Amersham Pharmacia Biotech/GE Healthcare) according to the manufacturer's protocol. For cytoplasmic and nuclear protein fraction isolation, the NE-PER™ Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Fisher Scientific) was used according to the manufacturer's protocol.

Silencing of gene expression by siRNA transfection

For *TUBB3* silencing, the DharmaFECT transfection reagent (Dharmacon/Thermo Fisher Scientific) was combined with ON-TARGET*plus* siRNA SMARTPool or ON-TARGET*plus* Nontargeting Pool (Dharmacon/Thermo Fisher Scientific) at a concentration of 25 nM for all cells except for OVCAR-3 cell variants when 12.5 nM was used. The siRNA pool was incubated in OPTI-MEM (Gibco/Thermo Fisher Scientific) for 20-30 min before addition to the cells. The cells were incubated at 37 °C for 48 h and then seeded for cell viability assays and gene and protein expression analyses.

Determination of the predictive and prognostic value of TUBB3 and TUBB

For analysis of the predictive and prognostic values of *TUBB3* and *TUBB*, ROC plotter [16] and KM plotter [17] online tools were used, respectively. The analysis was performed on all EOC samples (serous, endometrioid, clear cell), independent of the therapy received. The results were displayed as box and whisker plots, receiver operating characteristic (ROC) plots and/or Kaplan–Meier (KM) plots.

Determination of miR-200b and miR-200c promoter methylation

DNA was isolated from crude cell lysates using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The DNA concentration and purity were spectrophotometrically determined, and 500 ng of DNA from each sample was bisulfite converted and purified using an EZ DNA Methylation-Gold Kit (Zymo Research Europe, Freiburg, Germany) according to the manufacturer's protocol. Methylation-specific PCR for miR-200ab429 and miR-200c141 clusters was performed with 100 ng of bisulfite-converted DNA using the PyroMark PCR Kit (Qiagen). The conditions used are given in Table S3. Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems/Thermo Fisher Scientific) was used for cycling. The electrophoresis was run in a 3% agarose gel, and a 100-bp DNA ladder was used (New England Biolabs, Ipswich, USA). PCR for β-actin was performed according to Christmann et al. [18].

Transient transfection with miRNA mimic

DharmaFECT transfection reagent was combined with miRIDIAN microRNA Mimic 200c, 141 or scrambled negative control (Horizon Discovery/PerkinElmer, Waltham, USA) at a concentration of 12.5 or 25 nM and incubated in OPTI-MEM (Gibco/Thermo Fisher Scientific) for 20 min before addition to the MES-OV cell pair. Cells were incubated at 37 °C for 48 h and then seeded for the cell viability assay.

Inhibition of miRNA expression obtained by lentiviral particles carrying miRNA inhibitor

MES-OV and MES-OV CBP cells were infected with lentiviral particles containing scrambled control (sc) or hsa-miR-200c miRNA inhibitor (Inh200c), previously used and checked for their selectivity [9] (GeneCopoeia, Rockville, USA) in the presence of hexadimethrine bromide (Polybrene; Abbott Laboratories, Chicago, USA) and centrifuged for 90 min at 2000 rpm at 37 °C. After centrifugation, the cells were incubated at 37 °C for several days. The medium was changed once 24 h after transduction. The expression of mCherry in cells was checked 48 h after infection under a fluorescence microscope. MiRNA-inhibitor mCherry-positive cells (MES-OV sc, MES-OV CBP sc, and MES-OV Inh200c) were selected with hygromycin B (Sigma–Aldrich).

Determination of RAD50 localization in MES-OV CBP cells by confocal microscopy

The subcellular localization of RAD50 in MES-OV CBP cell lines with normal endogenous TUBB3 expression and with downregulated TUBB3 was analyzed by confocal microscopy. TUBB3 silencing was carried out as described above, and after 48 h, the cells were reseeded onto sterile glass microscope slides. Cells were grown on glass slides for 24 h and then treated, followed by immediate fixation with ice-cold methanol for 10 min at –20 °C. Samples were washed three times with PBS and blocked with 3% v/v bovine serum albumin in PBS for 30 min at room temperature. Staining was done with primary anti-TUBB3 antibody coupled with Alexa Fluor 488-tagged secondary antibody and primary anti-RAD50 antibody coupled with Alexa Fluor 555-tagged secondary antibody. Images were captured as single-plane confocal micrographs using a Leica TCS SP2 AOBS (Leica Microsystems, Wetzlar, Germany) confocal laser microscope system and analyzed with LAS X software (Leica Microsystems).

Statistical analysis

Viability assays and densitometry experiments data were analyzed by two-way ANOVA, and significance was determined between the resistant negative control compared with the parental negative control or to resistant cell line with silenced TUBB3. The experiments were performed either in quadruplicate or in duplicate and repeated at least twice. Real-time PCR data were analyzed by the unpaired Student's t test and expressed as the mean ± standard error of the mean. Experiments were performed in triplicate and repeated at least twice. Data were considered significant when P values were less than 0.05, and in the figures, these are designated * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ and **** = $P < 0.0001$.

Results

MES-OV CBP and SK-OV-3 CBP cells demonstrate reduced whole-cell and DNA platination and increased expression of several tubulin forms

To establish carboplatin-resistant cells, MES-OV and SK-OV-3 cells were treated with gradually increasing concentrations of CBP. The process resulted in an MES-OV CBP variant that was 2-fold and SK-OV-3 CBP cells that were 3.5-fold resistant to CBP (Fig. 1a; comparison of IC_{50} of CBP-resistant cells/IC_{50} of parental cells). Only MES-OV CBP was cross-resistant to paclitaxel (1.6 fold) (Fig. 1b). To elucidate the different responses of resistant cell variants to CBP in relation to the corresponding parental cell lines, the expression of several efflux pumps known to correlate with platinum drug resistance [7] was measured. Downregulation of *ABCC2* in MES-OV CBP (Fig. 1c) but upregulation in SK-OV-3 CBP (Fig. 1d) and downregulation of *ABCG2* mRNA levels in both cell lines (Fig. 1c and 1d) was observed. Furthermore, increased expression of ATP7A in MES-OV CBP cells (Fig. 1e) and ATP7B protein (Fig. 1f) in SK-OV-3 CBP was detected. Both CBP-resistant cell lines displayed decreased dose- (Fig. 1g and 1h) and time-dependent (Fig. S1a and S1b) levels of whole-cell platination and decreased levels of DNA platination (Fig. 1i and 1j). Since one of inhibitors of multidrug-resistant protein 2 (MRP2; *ABCC2*), MK571, sensitized resistant and parental SK-OV-3 cell lines to CBP in a similar manner, despite the increased level of *MRP2* expression observed in resistant cells (Fig. S1c), MRP2 protein could not be responsible for the detected differences in Pt uptake in SK-OV-3 parental and CBP-resistant cells. Taken together, obtained data implied that increased levels of the efflux pumps ATP7A and ATP7B could be a cause of the decreased levels of platinum detected in CBP-resistant cell lines.

Due to the combined therapy practice, we wanted to investigate why the acquired resistance to CBP resulted in cross-resistance to TAX in MES-OV CBP but not in SK-OV-3 CBP cells. MES-OV CBP-resistant variant displayed lower mRNA expression levels of P-glycoprotein (P-gp; ABCB1), efflux pump frequently described in the context of molecular mechanisms involved in TAX resistance [6, 10], but also in cDDP resistance [19], compared with their corresponding parental cell line, while in SK-OV-3 CBP, this downregulation was marginal (Fig. 1k and 1l). These results suggested other mechanisms were responsible for the cross-resistance of MES-OV CBP to TAX.

Due to the complex involvement of tubulin in TAX resistance [20], we next measured the constitutive expression level of several tubulin types and detected increased expression of tubulin γ and α in both CBP-resistant variants. Intriguingly, total tubulin β (pan β-tubulin; TUBB) that TAX directly binds [21, 22], resulting in the inhibition of microtubule disassembly [23], was significantly increased in MES-OV CBP compared with MES-OV cells (Fig. 1m). The same was not detected in SK-OV-3 CBP compared with SK-OV-3 cells (Fig. 1n). Although different TUBB isotypes could impact the cell response to TAX, we focused on the tubulin βIII isotype (TUBB3) whose increased expression is a known marker of poor clinical outcomes in advanced OC patients [1, 24].

Expression of TUBB3 was increased in both CBP-resistant cell models (Fig. 1m and 1n). Further, TUBB3 fibers of the MES-OV cell line pair appeared to branch out from the microtubule-organizing centers (MTOCs) in many directions, covering most of the nuclear surface. In the SK-OV-3 cell line pair a less branched TUBB3 net with fibers expanding from the MTOCs in parallel lines perinuclear toward cell poles was observed (Fig. S1d). In addition, the nuclei of MES-OV CBP cells appeared granulated, with notable variability in shape and size compared with the nuclei of MES-OV cells (Fig. S1e). These granulated nuclei may offer MES-OV CBP cells an advantage by promoting the functional connectivity of the nucleus with TUBB3 fibers. Moreover, there are visible TUBB3 fibers covering granulated cell nuclei (Fig. S1e). These observations need further investigation. Data available suggest that increased constitutive TUBB3 expression in MES-OV CBP cells could be a reason for the lower sensitivity toward TAX compared with parental MES-OV cells, while in the SK-OV-3 CBP variant it did not significantly affect the intensity of the TUBB signal compared with SK-OV-3 cells and seemed irrelevant for the TAX response (Fig. 1b and 1n). Moreover, it is worth mentioning that both SK-OV-3 and SK-OV-3 CBP cells lacked detectable levels of nonsoluble β-tubulin (TAX has been shown to bind to preassembled microtubules [25]), which might explain why they displayed similar sensitivity toward paclitaxel (Fig. S1f). Additionally, both CBP-resistant variants displayed a similar dynamic of TAX-induced tubulin polymerization as their respective parental cells (data not shown).

Depletion of TUBB3 sensitizes MES-OV CBP cells to paclitaxel as well as carboplatin

To confirm its relevance in the MES-OV CBP variant's response to paclitaxel, transient RNAi-based silencing of *TUBB3* was performed. Silencing *TUBB3* increased the sensitivity of the MES-OV CBP variant to TAX compared with the control RNAi, corroborating the involvement of TUBB3 in the response of MES-OV CBP cells to this drug (Fig. 2a). Interestingly, *TUBB3* silencing sensitized MES-OV CBP cells to CBP as well (Fig. 2b). Moreover, silencing *TUBB3* decreased the amount of TUBB to the level detected in the parental MES-OV cells. There was also no compensation for TUBB3 loss by other β-tubulin isotypes in MES-OV CBP cells, as the expression of class I, IIA, IVA, VI, and VIII β-tubulin did not change or even decreased after *TUBB3* silencing (Fig. 2c). Constitutively elevated TUBB3 levels did not cause cross-resistance to TAX in the SK-OV-3 CBP variant, and silencing *TUBB3* did not sensitize SK-OV-3 CBP cells to CBP (Fig. 2d). Although silencing *TUBB3* in the SK-OV-3 CBP variant decreased the amount of TUBB (Fig. 2e), as anticipated, this decrease was less significant than the effect observed in MES-OV CBP cells, pointing to compensation. Indeed, aside from a decrease in class VIII β-tubulin and a slight decrease in class IIA, the protein expression of β-tubulin isotypes I, IVA, and VI increased after RNAi-mediated attenuation of TUBB3 expression (Fig. 2e). Our next question was whether the effect of β-tubulin isotype compensation can occur in OC cells with the *vice versa* resistance.

Depletion of TUBB3 sensitizes paclitaxel-resistant and carboplatin cross-resistant MES-OV/TP in vitro and OVCAR-3/TP GFP cell line to both drugs in vitro and ex vivo

Cross-resistance of OC patients before or during therapy with CBP and TAX occurs in almost 30% of cases [3]. Therefore, we were further interested in the possible existence of the β-tubulin compensation phenomena in OC cells with acquired resistance to TAX. We used two previously described mesenchymal-like non-MDR paclitaxel-resistant variants, i.e., MES-OV/TP and OVCAR-3/TP GFP, which are cross-resistant to CBP [9]. Significant increase of TUBB in OVCAR-3/TP GFP cells and slightly increased expression in MES-OV/TP cells was detected. In OVCAR-3/TP GFP cells overexpressing TUBB3, most of the other isotypes were increased, while in MES-OV/TP only TUBB1 was slightly increased compared with the parental cells (Fig. 3b and 3d). Both TAX-resistant variants became statistically significantly sensitive to CBP (Fig. 3a and 3c; 2.1-fold and 2.8-fold) and TAX treatments (Fig. S2a and S2b) upon silencing of *TUBB3*. Moreover, *TUBB3* silencing was not compensated by overexpression of other β-tubulin isotypes (Fig. 3b and 3d). These results indicated that MES-OV TP and OVCAR-3/TP GFP cells, although with acquired resistance to TAX, shared a similar response pattern with MES-OV CBP cells with acquired resistance to CBP.

We additionally explored ascites collected from randomly selected nude mice with peritoneal injected OVCAR-3/TP GFP cells used for experiments shown in Fig. 5 of Moisan et al. [13]. Ascites from nontreated and paclitaxel-treated mice were sorted to isolate GFP-positive OVCAR-3/TP cells for further characterization, named #6 (untreated OVCAR-3/TP GFP mice) and #8 (TAX-treated OVCAR-3/TP GFP mice) (Fig. 3e). Comparative analysis showed that both #6 and #8 cell lines were very similar to the original OVCAR-3/TP GFP cells regarding expression of *CDH1* and *FN1* (Fig. S2c) and most of the miRNA-200 family members (Fig. S2d), markers previously used for the characterization of the OVCAR-3/TP GFP cell model [9]. Surprisingly, the #8 cell line showed significantly enhanced sensitivity to TAX (Fig. S2e), CBP (Fig. 3f), and cDDP (Fig. S2f) compared with the original OVCAR-3/TP GFP and #6 cell lines. We hypothesized that this hypersensitivity might be due to altered expression of β-tubulin isotypes in OVCAR-3/TP GFP cells upon TAX treatment *in vivo*. Thus, tumor sensitivity toward anticancer therapeutics could be connected with the decreased expression levels of β-tubulin isotypes. To test this hypothesis, the expression of TUBB and β-tubulin isotypes was measured. Several β-tubulin isotypes, as well as the amount of TUBB, was reduced in #8 cells compared with #6 cells (Fig. 3g). These data suggested that the composition of β-tubulin isotypes together with TUBB3 influence the cell response to both TAX and platinum drug therapy *ex vivo*.

TUBB and TUBB3 gene expression signatures in ovarian cancer outperform the predictive and prognostic values of each gene separately

The observed correlation of *TUBB* and *TUBB3* expression with the specific cellular response to the drug treatment *in vitro* and *ex vivo* led us to hypothesize that patients who did not respond to the combination therapy (nonresponders) compared with those who responded well (responders) could have increased gene expression of both TUBB and TUBB3 and shorter progression-free survival (PFS). To test this hypothesis, ROC and KM plotter tools were used. The obtained data showed that the expression of *TUBB3*, as well

as *TUBB3/TUBB* signature, was significantly increased in patients with epithelial OC (serous, endometrioid, clear cell) who experienced progression of the disease six months after therapy (Fig. 4a). However, 12 months after therapy, only *TUBB3/TUBB* signature was significant (Fig. 4b). Interestingly, when the prognostic value of both genes was analyzed, patients with high *TUBB3* had better PFS, while those with high *TUBB3*/*TUBB* signatures had shorter survival (Fig. 4c). The obtained data suggested that *TUBB* expression, along with *TUBB3*, should be observed regarding the prediction of the therapy response and the clinical prognosis of OC patients. Similar results were observed when only patients with high-grade serous OC in stages 2, 3, and 4 were analyzed (Fig. S3a-S3c). This finding confirmed the data collected from explored cell models.

MiRNA-200c mediates carboplatin-induced upregulation of TUBB3 in MES-OV CBP cells

It is known that TUBB3 expression is regulated by miRNA-200 family members [26] and that the expression of miR-200c correlates inversely with TUBB3 expression in OC cell lines resistant to TAX [9]. However possible interplay between CBP-induced stress and TUBB3 expression has not been studied. The expression pattern of miRNA-200 family members differed between the two CBP-resistant cell pair models (Fig. 5a). Due to the possible epigenetic regulation of miR-200 family members expression, we checked the methylation status of their promoters. Unmethylated (U) DNA was observed in the MES-OV cell line for both miR-200c and miR-200b promotors, while only the methylated form (M) was detected in the MES-OV CBP cells (Fig. 5b left) corresponding with the downregulated expression of all five members of the miR-200 family in MES-OV CBP cells. However, there was no detectable difference in the methylation status of the two promoters between SK-OV-3 and SK-OV-3 CBP cells (Fig. 5b right). These data suggested that long-term exposure of MES-OV cells to CBP caused molecular changes linked with epigenetic regulation, in this case, changes in the methylation status of miR-200 promoters. Further focusing on the miR-200c, we treated MES-OV CBP cells with two different concentrations of the DNA methylation inhibitor 5-aza which resulted in upregulated expression of miR-200c, supporting the hypothesis that altered epigenetic modulation caused by repeated CBP treatment might be responsible for its downregulation. Moreover, under the same experimental conditions, *TUBB3* expression was downregulated (Fig. 5c), supporting the proposed model of CBP-induced downregulation of miR-200c and consequential overexpression of *TUBB3*. In addition, 5-aza pretreatment sensitized MES-OV CBP cells to CBP by 1.3-fold (comparison of IC_{50} value of 5-aza+CBP/IC_{50} of CBP-only treated cells; Fig. 5d). Further, transient transfection of specific miR-200c mimics into resistant cells sensitized them to CBP treatment compared with the nontarget control (Fig. 5e) (i.e., 1.1-fold in the case of 25 nM mimic, 1.2-fold for 12.5 mimic; comparison of IC_{50} value of miR-200c mimic/IC_{50} of nontarget control). Moreover, exogenous overexpression of miR-141, which is regulated by the same promoter as miR-200c, did not influence the sensitivity of MES-OV CBP to CBP, either transfected individually or as part of the mimic pool (Fig. 5e). The minimal shift in sensitivity after transient transfection was not unexpected based on previous experience [9], when stable transfection was needed for a more significant effect. Consequently, transduction of MES-OV cells with lentiviral particles carrying either empty or miR-200c inhibitor construct was performed and MES-OV sc, MES-OV Inh200c, and MES-OV CBP sc were established. Downregulated expression of miR-200c in the MES-OV Inh200c compared with MES-OV sc cells was confirmed by RT–qPCR (Fig. 5f). Inhibition of miR-200c in MES-OV cells rendered them 2.3-fold (comparison of IC_{50} value of Inh200c/IC_{50} of empty vector control) less sensitive to CBP (Fig. 5g) and 1.4-fold less sensitive to TAX (Fig. S4). Most importantly, decreased constitutive expression of miR-200c was accompanied by elevated expression of TUBB3 and TUBB in MES-OV Inh200c compared with MES-OV sc cells (Fig. 5h). This finding shows for the first time that the miR-200c/TUBB3 axis, which is triggered by CBP-induced constitutive changes in the methylation of the miR-200c promoter, is part of the cellular stress response to CBP.

TUBB3 silencing impacts the DNA damage response and increases cell platination

We were intrigued that the depletion of TUBB3 in MES-OV CBP cells sensitized them to CBP (Fig. 2b). To investigate the possible relationship between the acquired expression of TUBB3 in CBP resistant cells and DNA damage response, we asked could *TUBB3* silencing prolong the effect of DNA damage caused by CBP. We measured the expression of γH2AX over time, an indicator of DNA damage [27], upon a four-hour CBP exposure and under the experimental conditions where the viability of drug resistant cells was not compromised (data not shown). As shown in Fig. 6a, in MES-OV CBP si(-)-transfected cells the induced level of γH2AX decreased in time, while in MES-OV CBP cells with silenced *TUBB3* γH2AX levels remained high, and were comparable with the γH2AX signal detected in parental/CBP-sensitive MES-OV si(-)-transfected cells. This result confirmed the hypothesis that *TUBB3* silencing could impede DNA damage response triggered by CBP treatment.

As it was shown previously that hindered microtubule dynamics can compromise DNA repair proteins trafficking [28] we further hypothesized that TUBB3 could be involved in the trafficking of DNA damage response (DDR) proteins from the cytoplasm to the nucleus as part of the response to CBP-induced stress in MES-OV CBP cells. We measured the expression of several proteins known to be involved in the recognition of double-strand breaks (RAD50, p95/NBS1, MRE11, XRCC5) [29, 30], choosing them from a larger group initially considered based on their performance on immunoblots (Fig. 6b left). Silencing *TUBB3* in MES-OV CBP cells in combination with CBP stress resulted in the reduction of RAD50 and XRCC5 levels in the nucleus compared with si(-)-transfected cells, and a consequential increase in the cytoplasmic fraction (Fig. 6b right), indicating impaired trafficking of both proteins from the cytoplasm to the nucleus. Silencing *TUBB3* did not impact the trafficking of MRE11 and p95/NBS1.

To visualize DNA repair-related proteins trafficking, confocal microscopy in CBP-resistant MES-OV cells after *TUBB3* silencing was performed and RAD50 protein was observed due to its immunofluorescence performance. In the untreated MES-OV si(-)-transfected cells, RAD50 signals were detectable within cell nuclei and on the edges of cell membranes, while upon CBP treatment, signals were more prominent within the nucleus (Fig. 6c and S5). Due to the equimolar concentration of CBP used, the impact of treatment on RAD50 nuclear accumulation in MES-OV CBP si(-) cells was less significant compared to MES-OV si(-). However, *TUBB3* silencing

in these cells led to the reduction of RAD50 nuclear signal and its accumulation in the cytoplasm, confirming the results obtained by Western blot. We can assume that hindered microtubule integrity, caused by *TUBB3* silencing, disrupted RAD50 trafficking from cytoplasm to the nucleus.

In addition, our data showed that upon silencing *TUBB3* in MES-OV CBP cell lines, the whole-cell platination level increased in a dose- and time-dependent manner (Fig. 6d). Building on these features, our results suggest that the silencing of *TUBB3* could be responsible for the impaired vesicle-mediated export of sequestered CBP and consequentially increased whole-cell platination. However, this possibility requires further examination.

Discussion

The combination of platinum drugs and taxanes is commonly used to treat patients with OC [31]. However, the development of therapy resistance by approximately 30% of OC patients, even though they may respond well at the beginning, contributes to poor long-term therapy outcomes [3]. The variations in treatment response underscore the need for a more detailed molecular characterization of OC to identify beneficial treatment options for individual patients. Evidence supports the use of DNA repair proteins (ERCC1, BRCA1/2) as markers for the platinum response [32]; however, there are also conflicting data [33, 34]. In addition, TUBB3 has been indicated as a predictive biomarker of taxane responsiveness in clinical settings [35, 36]. However, there is also contradicting evidence showing that TUBB3 overexpression has a negligible effect on tumor cell sensitivity to paclitaxel [37], which we also confirmed herein by showing that increased expression of TUBB3 in SK-OV-3 CBP cell line compared with SK-OV-3 cells did not impact TAX sensitivity. Therefore, additional evaluation [38] and data are needed to identify meaningful biomarkers for therapy response prediction.

We generated two CBP-resistant mesenchymal-like variants, i.e., MES-OV CBP and SK-OV-3 CBP from the parental OC cell lines MES-OV and SK-OV-3. Unlike MES-OV CBP, the SK-OV-3 CBP variant did not exhibit cross-resistance to TAX. We further showed that both resistant variants accumulated less platinum and that increased TUBB3 had an impact on the response of MES-OV CBP to TAX, molecular mechanisms that are mainly explored in the context of CBP (influx and efflux cell capacity) and TAX (TUBB3 expression) resistance [6]. Taken together, these CBP-selected cell lines, one non-MDR cell line cross-resistant to TAX (MES-OV CBP) and one sensitive to TAX (SK-OV-3 CBP), represent very interesting and clinically relevant models of drug resistance [3]. However, the question is whether these mechanisms of resistance cross paths and depend on each other. Considering that a combination of platinum and taxane drugs is used in the treatment of not only ovarian but also a variety of other cancers, it is crucial to address this question. Phenomenon of *TUBB3* silencing impacting the cell response to CBP, observed here in MES-OV CBP as well, has been described previously in human non-small lung cancer cells [39], but it was hypothesized that TUBB3, especially mitochondrial tubulin, may act as a survival factor to rescue tumor cells from death signals triggered by chemotherapeutic agents. Compensation effect or β-tubulin isotype balance, that we propose with the SK-OV-3 CBP model, is known to be involved in the cellular stress response to taxane, colchicine, and vinca drugs [40, 41] but not in the context of platinum drugs.

Our observation that TUBB3 expression is induced in OC cells with acquired resistance to CBP and that the lack of β-tubulin isotype compensation after *TUBB3* silencing impacts the cellular response to CBP encouraged us to explore whether these intertwined processes also occur in paclitaxel-selected cells. Previously, OC cell variants stably resistant to TAX and cross-resistant to CBP MES-OV TP and OVCAR-3/TP GFP were developed, with increased constitutive expression level of TUBB3 compared with the parental cell line [9, 13, 42]. Silencing *TUBB3* resulted in sensitization of variants to CBP accompanied with a lack of β-tubulin isotype compensation. This novel finding supported the hypothesis that the phenomenon observed in CBP-resistant cells can also occur in cells with a reverse resistance status: cells with acquired resistance to TAX and cross-resistance to CBP. The importance of understanding the β-tubulin isotype balance is emphasized already as an interesting area of exploration [43] due to their role in microtubule dynamics as well as in oncogenic changes that provide a survival or proliferative advantage to cancer cells within the tumor microenvironment and during metastatic processes [41]. Moreover, this β-tubulin isotype balance was shown to occur *in vivo*, as TUBB3$^{-/-}$ mice compensate for the lack of TUBB3 with other β-tubulin isotypes to have wild-type TUBB levels [44]. Interestingly, data obtained regarding the sensitivity of #6 and #8 variants, isolated from TAX-untreated and treated mice, to TAX, CBP, and cDDP showed that the #8 variant became more sensitive to all drugs compared with OVCAR-3/TP GFP and #6 cells, with decreased expression of TUBB, and other isotypes. This finding underlines that even *in vivo*, the balance of β-tubulin isotypes seems essential in the tumor cell response to anticancer therapeutics.

It is important to keep in mind that the development of resistance through *in vitro* dosing may not fully recapitulate the various mechanisms of resistance that could be imparted through the patient's tumor microenvironment [45]. Nevertheless, according to OC clinical data analysis, there was a correlation between TUBB, TUBB3, and a response to therapy, which should not be overlooked. *TUBB* and *TUBB3* gene expression signature in OC and serous OC outperformed the predictive and prognostic values of *TUBB3* gene alone.

Finally, the types of changes that were induced by long-term and repeated exposure of cells to CBP and their link to TUBB3 remain unanswered questions. The miRNA-200 family members, regulators of epithelial-mesenchymal transition, play an important role in cell migration, invasion, and drug resistance [46] and regulate the expression of different proteins, including TUBB3 [47]. Although we noticed that the presence of compensation phenomena correlated with the expression pattern of miRNA-200 family members, at least in OC cell lines, the molecular mechanism underlying the possible TUBB isotype compensation regulation by miR-200 family is not known. The upregulation of miR-200c has been reported to contribute to cDDP resistance in biliary tract cancer [48], and was correlated with cDDP resistance in esophageal cancer patients via induction of Akt signaling and inhibition of PP2A (protein phosphatase 2A) [49]. We showed that in MES-OV CBP cells, exposure to CBP induced constitutive changes in miR-200c

expression, accompanied by alterations in epigenetic regulation. We further showed that miR-200c regulated TUBB3 expression and impacted the cellular response to CBP and TAX, determining CBP-induced miR-200c/TUBB3 axis for the first time. The observation that miR-200c was upregulated in SK-OV-3 CBP compared with SK-OV-3 cells, accompanied by the same methylation status of the miR-200c promoter, indicated that different regulatory mechanisms probably developed during the establishment of this model. Additional research is needed to understand the correlation between miR-200 family members' expression pattern, downstream regulations and eventually cell response to different drugs.

Most studies investigating TUBB3 in the context of the cellular stress response and its relevance in the development of tumor cell resistance have focused on the stress caused by microtubule toxins [28, 29]. To the best of our knowledge, only one study has shown that silencing TUBB3 sensitizes non-small cell lung cancer H460 cells to the DNA-damaging agents cisplatin, doxorubicin, and etoposide [30]. The obtained data triggered our interest in exploring the role of TUBB3 in the cell response to CBP stress in the MES-OV CBP variant, where the lack of TUBB3 was not compensated by elevated expression of another β isotype. Carboplatin exerts its cytotoxic activity, among others, through reaction with the DNA, creating damage in the form of intrastrand and interstrand cross-links and single-nucleotide damage to guanine [4]. Interstrand cross-links (ICL) are the most toxic and their repair requires combination of processes, among others translation synthesis, nucleotide-excision repair and homologous recombination. The first step of ICL repair involves recognition of the damage and incision on DNA near the crosslink. Incision at the ICL can occur before or after bypass, leaving double-strand breaks (DSB) subject to homologous recombination. DSBs formation is followed by the phosphorylation of the histone H2AX (γH2AX), the sensor of DSBs required for recruiting and localizing DNA repair proteins [27], and binding of two multiprotein complexes - MRE11/RAD50/NBS1 (MRN) and XRCC6/XRCC5 (Ku86) to the DNA ends [50]. Poruchynsky et al. showed that microtubules are important in the trafficking of proteins involved in processing DNA damage [28]. They further showed that proteins involved in the repair of DNA damage associate with dynein, cytoskeletal motor proteins that move along microtubules in cells [51], and with microtubules. Here, we showed for the first time that in a cell line with acquired resistance to CBP, *TUBB3* silencing prolongs the retention of γH2AX indicating less efficient CBP-induced DNA damage response. Further, we showed for the first time that TUBB3 was involved in CBP-induced trafficking of the DNA repair-related proteins RAD50 and XRCC5 from the cytoplasm into the nucleus and visualized that impact by following the intracellular localization of RAD50 in MES-OV CBP cell lines upon CBP treatment. Related to that we are speculating that the observed granulated nuclei together with increased tubulin γ [52] may offer MES-OV CBP cells an advantage by promoting the functional connectivity of the nucleus with TUBB3 fibers [53] and possibly more efficient protein trafficking. This would be interesting to follow in the future. It is known that platinum drugs are sequestered into vesicular structures of the lysosomal, Golgi, and secretory compartments [54] and that microtubules are involved in vesicular transport [6, 55]. In this way, vesicles and exosomes can facilitate drug sequestration by directly loading and exporting antitumor agents from cancer cells [56]. ATP7A, e.g., is redistributed by microtubules to different vesicular compartments that traffic from the trans-Golgi network to the corresponding domain of the cell membrane [57]. Our data showed that silencing *TUBB3* resulted in increased whole-cell platination in the resistant MES-OV CBP cell line, which also showed a higher level of ATP7A protein than the parental cells. These data indicated to the possible involvement of TUBB3 upregulation in vesicular trafficking as well and that this trafficking could play a part in the decreased CBP sensitivity of the MES-OV CBP cell line.

In summary, by using several cell models differing in their origin, type, and protocol of acquired resistance development, we found that increased constitutive expression of TUBB3 plays distinct roles in the cellular stress response and resistance to drugs, depending on whether replacement by other tubulin isoforms is possible (Fig. 7). This phenomenon underlines why TUBB3 is a useful predictive marker for treatment efficacy in some cases, while in other cases, it is not. We hypothesize that cells exhibiting a downregulation of miRNA-200 family members, especially miR-200c, lack the mechanism(s) to compensate for β-tubulin isotypes, unlike the cells in which downregulation of all members is not detected. These divergent effects should be further investigated.

Statements and Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Authors Contributions M.P.K., V.T. and A.B. performed the experiments, with assistance from J.K., G.E.D., A.S., D.S.P., S.D., N.B., M.T.C., P.H.; M.P.K., V.T. and A.B. analyzed and visualized the data; B.I.S. provided resources for the beginning of the MES-OV CBP cell line establishment. G.E.D and B.I.S were involved in language editing; A.B. conceptualized and supervised the project, acquired the funding, wrote original draft, and together with M.P.K and V.T. reviewed and edited the manuscript.

Ethics approval

The Administrative Panel on Laboratory Animal Care (APLAC) of Stanford University, USA approved all protocols in compliance with the Guide for the Care and Use of Laboratory Animals. The laboratory animal care program at Stanford is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

Consent for publication
Not applicable.

Availability of data and material
Not applicable.

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Figure Legends

Fig. 1 Non-MDR MES-OV CBP cells are cross-resistant to TAX, unlike SK-OV-3 CBP cells (a)(b) The cells were treated with different concentrations of CBP and TAX for 72 h. Cell viability was measured by the Alamar blue assay. All data are expressed as the average percentage of viability values relative to an untreated control ± SD. (c)(d) The constitutive expression of *ABCC2* (*MRP2*) and *ABCG2* (*BCRP*) was measured in cells by RT–qPCR 48 h after seeding. *GAPDH* was used as an internal loading control for normalization. (e)(f) Constitutive expression of ATP7A and ATP7B proteins in parental and resistant cell lines was measured by Western blot analysis 48 h after seeding. Densitometry results represent protein values normalized to the β-actin protein level. The values of parental cells were set as 1. A representative blot of two independent experiments is shown. (g)(h) Twenty-four hours after seeding, the cells were treated with 40, 160 and 320 µM CBP for 4 h. Whole-cell platination levels were determined by HR ICP–MS. (i)(j) Twenty-four hours after seeding, cells were treated with 320 and 640 µM CBP for 6 h. DNA platination levels was determined by HR ICP–MS. (k)(l) The constitutive mRNA expression of *ABCB1* was measured in cells by quantitative real-time PCR 48 h after seeding. *GAPDH* was used as an internal loading control for normalization. Fold change in expression between resistant compared with parental cell lines was calculated as $2^{-\Delta\Delta Ct}$. The parental line was set as 1. (m)(n) Constitutive expression of tubulin forms in parental and resistant cell lines was measured by Western blot analysis 48 h after seeding. Densitometry results represent protein values normalized to total ERK1/2. The values of the parental cells were set as 1. A representative blot of three independent experiments is shown. Significance was determined between the resistant compared with the parental cell line. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Fig. 2 TUBB3 silencing differentially affects the sensitivity to CBP and the expression of other TUBB isotypes in MES-OV CBP and SK-OV-3 CBP cells Twenty-four hours after seeding, the cells were transfected with 25 nM si(-) or si(*TUBB3*) for 48 h. (a)(b)(d) Transfected cells were seeded on 96-well plates and treated with different concentrations of TAX and CBP. Cell viability was measured after 120 h with an Alamar blue assay. (c)(e) Expression of TUBB isotypes I, IIA, III, IVA, VI, VIII, and TUBB was measured by Western blotting. Densitometry results represent tubulin values normalized to the total ERK1/2. Tubulin values of parental cells (MES-OV si(-) or SK-OV-3 si(-)) were set as 1. A representative blot of two independent experiments is shown. Significance was determined between the resistant negative control compared with the parental negative control or with the resistant cell line with silenced TUBB3 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Fig. 3 TUBB3 depletion affects the drug-sensitivity of TAX-resistant cells *in vitro* and OVCAR-3/TP GFP cells *in vitro and ex vivo* (a)(c) Twenty-four hours after seeding, MES-OV, MES-OV TP, OVCAR-3 GFP and OVCAR-3/TP GFP cells were transfected with 25 or 12.5 nM si(-), respectively or with si(*TUBB3*) for 48 h. Cells were then seeded on 96-well plates and treated with different concentrations of CBP. Cell viability was measured after 120 h with an Alamar blue assay. (b)(d) Expression of TUBB isotypes I, IIA, III, IVA, VI, VIII, and TUBB was measured by Western blotting. Densitometry results represent tubulin values normalized to the total ERK1/2. Tubulin values of parental cells (MES-OV /OVCAR-3 GFP si(-)) were set as 1. A representative blot of two independent experiments is shown. (e) OVCAR-3/TP GFP cells were injected in nude mice which are treated accordingly to Fig. 5 in Moisan et al. [13]. The #6 and #8 cell lines were obtained as decribed in Material and Methods. Graphic created with BioRender.com. (f) The cells were treated with different concentrations of CBP for 72 h. Cell viability was measured by the Alamar blue assay. All data are expressed as the average percentage of viability values relative to an untreated control ± SD. (g) Expression of TUBB isotypes I, IIA, III, IVA, VI, VIII, and TUBB was measured by Western blotting. ERK1/2 was used as a loading control. Densitometry results represent tubulin values normalized to the total ERK1/2. Tubulin values of chemotherapy-naive cells (#6) were set as 1. Significance was determined between the resistant negative control compared with the parental negative control or with the resistant cell line with silenced TUBB3 or between OVCAR-3/TP GFP compared to #6 and #8 cells (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Fig. 4 Importance of TUBB3 and TUBB confirmed on clinical data The expression of *TUBB* and *TUBB3* (a) six months after therapy of 1226 and (b) twelve months after therapy of 1174 serous, endometroid, and clear cell OC patients' data were analyzed using the receiver operating characteristic (ROC) plotter online tool. Patients were divided into nonresponder and responder groups. Predictive values of each gene separately and in combination were analyzed. The two cohorts, nonresponders and responders, were compared by the Mann–Whitney and ROC tests. (c) To determine the prognostic values of each gene separately and in combination, data from 1435 patients were analyzed by the KM plotter online tool and are shown as PFS. The hazard ratio (HR) and log-rank P value were calculated and are displayed. N, number of patients; 95% CI, 95% confidence interval; P bold, statistically significant.

Fig. 5 Epigenetically regulated microRNA-200c modulates sensitivity of MES-OV CBP cells to CBP via TUBB3 (a) The constitutive expression of miR-200 family members was determined by RT–qPCR 48 h after cell seeding, using *RNU6* as an internal loading control. Fold change in expression between resistant compared with parental cell lines was calculated as $2^{-\Delta\Delta Ct}$. The parental line was set as 1. (b) DNA isolated from MES-OV and SK-OV-3 cell pairs was bisulfite converted, and methylation-specific PCR for miR-200b and miR-200c clusters was performed. (c) MES-OV CBP cells were treated with 1 or 5 µM 5-aza for 72 h (fresh medium and drug added every 24 h). The expression of miR-200c and *TUBB3* was determined by RT–qPCR using *RNU6* (left) or *GAPDH* (right) as an internal loading control. Fold change in expression between the treated compared with the untreated MES-OV CBP cell line was calculated as $2^{-\Delta\Delta Ct}$. Untreated cells were set as 1. (d) After 72 h of pretreatment with 1 µM or 5 µM 5-aza, cells were treated with different concentrations of CBP for 72 h. Cells were then fixed with cold methanol and stained with crystal violet. ImageJ was

used to measure the intensity of color and area under the curve (AUC), proportional to cell proliferation. Untreated controls were set as 100% to calculate % of AUC. (e) Twenty-four hours after seeding, cells were transfected for 48 h with 25 nM mimic(-), mimic(miR-200c) or mimic(miR-141), or with the mimic pool (12.5 nM of each). Twenty-four hours after reseeding cells were treated with different concentrations of CBP. Cell viability was measured after 120 h with an Alamar blue assay. (f) The expression of miR-200c in MES-OV sc, MES-OV Inh200c and MES-OV CBP sc cells was checked by RT–qPCR using *RNU6* as an internal loading control. The fold change in expression was calculated as $2^{-\Delta\Delta Ct}$. MES-OV sc cells were set as 1. (g) Twenty-four hours after seeding, MES-OV sc, MES-OV Inh200c and MES-OV CBP sc cells were seeded on a 96-well plate and treated with different concentrations of CBP. Cell viability was measured after 72 h with the Alamar blue assay. (h) The expression of TUBB3 and TUBB was measured by Western blotting. Densitometry results represent TUBB3 and TUBB values normalized to GAPDH. Tubulin values of MES-OV sc were set as 1. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Fig. 6 TUBB3 silencing affects the trafficking of DNA repair protein in MES-OV cells Twenty-four hours after seeding, cells were transfected with 25 nM si(-) or si(*TUBB3*) for 48 h. (a) Cells were reseeded and day after treated with 320 µM CBP for 4 h. Cells were collected 0, 24, 48 and 72 h after the treatment. The expression of γH2AX and H2AX was determined by Western blot. A representative Western blot of two independent experiments is shown. (b) Transfected cells were replated in duplicate. Twenty-four hours later, each sample was either treated with 60 µM CBP for 4 h or left untreated as a control. Cytoplasmic (CF) and nuclear (NF) fractions were isolated, and the expression of RAD50, MRE11, p95/NBS1 andXRCC5 was measured by Western blotting. The same percentage of CF and NF volume (25%) was loaded on the gel (8%). Actin, PARP-1 and PCNA were used as fractionation controls. A representative Western blot of two independent experiments is shown (left). The percentage of RAD50 and XRCC5 in CF is indicated on the right and calculated as [CF/(CF+NF)] x 100. (c) Transfected cells were replated on glass slides. Twenty-four hours after seeding, the cells were either treated with 60 µM CBP for 4 h or left untreated as a control. Cells were fixed, permeabilized, and stained with anti-TUBB3 antibody (green) and anti-RAD50 antibody (red). Nuclei were stained with DAPI (blue). Panels show single-plane confocal micrographs of si(-) and si(*TUBB3*) transfected cells. The merged images are shown in the bottom row. Scale bar = 10 µm. (d) Transfected cells were replated in duplicate. Twenty-four hours after seeding, cells were treated with either 40, 160, or 320 µM CBP for 2 h for dose–response results or with 60 µM CBP for 2 and 4 h for time-response results. Whole-cell platination levels were determined by HR ICP–MS. Significance was determined between the resistant negative control compared with the parental negative control or with the resistant cell line with si(*TUBB3*) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Fig. 7 Hypothetical model of the role of TUBB3 in acquired resistance to CBP Acquired resistance to CBP in two OC cell models resulted in increased TUBB3 expression but in different constitutive levels of TUBB. Upregulation of TUBB3 disrupts microtubule dynamics and has been linked to TAX resistance [8]. On the other hand, silencing of TUBB3 sensitized cells to CBP, where compensation of β-tubulin isotypes was not detected. The involvement of microtubules in the trafficking of DNA repair proteins [28] and vesicles [54, 55] could explain the observed effect. It remains unclear how β-tubulin isotype compensation is regulated. Thus, we still do not understand why this phenomenon is present in some, but not all, cell models. Summarizing the data, we suggest that acquired resistance to CBP results in increased TUBB3 expression, which in cells lacking β-tubulin isotype compensation is involved in the cell response toward CBP and TAX.